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Employment of Somatic Embryogenesis as a Tool for Rescuing Imperiled *Narcissus tazetta* L. Growing Wild in Jordanian Environment.

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Abstract

Somatic embryogenesis was used as a tool for micropropagation of wild *Narcissus tazetta* plants exposing to overcollection and rapidly changed environmental conditions. In the growth regulators experiment, somatic embryogenesis was successfully induced in all treatments except for explants grown in the control treatment (Murashig and Skoog (MS) hormone-free medium). Meanwhile, the highest value for the number of somatic embryos/ callus segment (441) was obtained into MS media supplemented with 0.2 mg l^{-1} 6-(gamma, gamma- Dimethylallylamino) purine (2iP) under dark conditions. Moreover, sucrose at 30g L^{-1} was the best sugar source resulting in higher number of somatic embryos compared to the other sugar treatments. The highest shoot development rate from somatic embryos was (191.99/ non-embryonic calli segment) recorded in cultures grown on MS media plus 0.5 mg l^{-1} 2iP. Maximum bulbet size (1.6 cm diameter) was recorded in plantlets kept onto hormone-free MS hormone free media for 6 weeks before acclimatization, while less durations resulted in smaller bulbet size. Well developed *in vitro* plantlets were acclimatized successfully with high survival percentage of (95%). The acclimatized plants were normal and did not show any morphological abnormalities.

Keywords: Callus, Embryogenesis, *Narcissus tazetta*, Somatic embryo.

1. Introduction

Narcissus tazetta is a wild ornamental plant that grows naturally in the hills and mountainous rocky grounds of Jordan. It is known by the local community as (Narjes Baladi). It has an important ornamental value due to its white-cream with orange crown flowers and its distinctive odor (Al-Eisawi, 1998). In addition, many wild plants in Jordan had been reported for their medicinal activities (Alenizi *et al*., 2020; Al Qudah, 2020; Tahtamouni *et al*., 2016). Also, *Narcissus tazetta* was reported recently in many research articles as a natural source of galantamine (GAL) which has been prescribed for treatment of Alzheimer's disease (Bores and Kosley, 1996; Khonakdari *et al*., 2020). Because of overexploitation through uprooting and continuous removal of the plants, natural habitat destruction, climate change, and increasing demands on this plant for both ornamental and medicinal values (Alenizi *et al*., 2020), the wild *N. tazetta* populations in Jordan are exposed to extinction (RBG, 2016). Propagation of this valuable genetic resource is imperative for its survival and continuity. Unfortunately, *N. tazetta* propagation through vegetative methods by chipping and twin scales is not efficient due to its slow propagation (Stone, 1973; Stone *et al*., 1977). Propagation of *N. tazetta* can help in conserving this valuable plant from extinction. In vitro development techniques including somatic embryogenesis are important and easy methods of vegetative multiplication, and they have the advantage of rapid multiplication (Shibli *et al*., 2012). High numbers of genetically uniform plants can be cultured from a single plant by using those techniques (Al Qudah *et al*., 2011; Shibli *et al*., 2018). Somatic embryogenesis is one of the basic tools widely used in plant biotechnology and *in vitro* development research. It is useful for micropropagation and production of transgenic plants, which can be used for producing fully transformed plants after mutagenesis or gene transfer (Mostafa *et al*. 2010). Somatic embryos can be produced in high frequencies, but maturation and plant development are still a difficult task, requiring optimization of medium and environmental conditions (Kumar *et al*., 2013; Shibli *et al*., 2012). In reviewing the literature, until now, there were no reports on the in vitro development via somatic embryogenesis of the valuable wild Jordanian *N. tazzeta* L. Thus, this study was carried out to develop a protocol for in vitro massive propagation via somatic embryogenesis and ex vitro acclimatization of *N. tazetta* L, hoping that this approach might contribute to its sustainability.

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2. Materials and methods

2.1. Plant material

Bulbs of *Narcissus tazetta* L. were collected from Ajloun- Kufranja during December of 2014 (N 32.25006°, E 35,652336°) at 210 m above sea level (Fig. 1). The experiments were held at the plant biotechnology laboratories at Hamdi Mango Center/ Faculty of Agriculture, The University of Jordan, (Amman-Jordan).

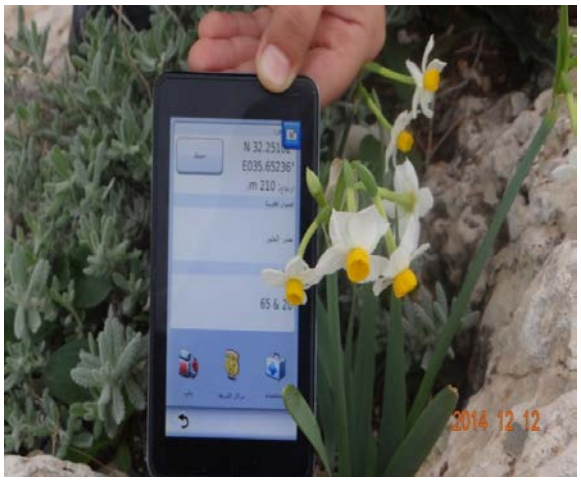

Figure 1. N. tazetta flowering plant in the wild at Ajloun-Kufranja during December, 2014 (N 32.25006°, E 35,652336°) at 210 m above sea level.

2.2. Callus and Embryos induction

To induce callus and embryos formation, sterilized segments of inner bulb scales of *Narcissus tazetta* were grown on (Murashig and Skoog, 1962) (MS) media premix (Duchefa Biochemiea: Murashige and Skoog media plus vitamins; Duchefa-Postbus 809,2003 RV Haarlem, Netherlands) supplemented with different concentrations (0.5, 1.0 or 2.0 mg/L) of 6-Benzylaminopurine (BAP) plus 0.1, 0.4, 1.0, 2.0 mg/L of 1-Naphthaleneacetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D). Next, the inner bulb scales were transferred to the growth room which consisted of the following physical conditions (the growth room temperature was 24±1°C under a 16/8 (light/dark) and 45–50 µmol / m^2s irradiance or to full dark conditions) and maintained for five weeks. Data was taken for callus formation percentage in each treatment. Data showed that 1.0 mg l^{-1} of (BAP) with 2.0 mg l^{-1} (2,4-D) (Figure 2) was found to be the best formula for callus initiation (data is still under publication).

Next, different embryos induction media with different types and levels of growth regulators or carbon sources (sugars) were investigated. In the first experiment, different concentrations (0.0, 0.2, 0.5, 1.0, 1.5 or 2.0 mg/ L) of growth regulators; (2iP, BAP, or Kinetin (KIN) were used. In the second experiment, different sugar types (sucrose, glucose or fructose) and levels (0, 10, 30, 40, 50 g/L) were added to MS callus induction media which contained 1.0 mg l-1 of (BAP) plus 2.0 mg l-1 (2,4-D). Each treatment in both experiments was replicated five (Petri dishes), each with 4 callus segments (250 mg). Cultures were maintained in dark undergrowth room conditions where growth room temperature was 24 ± 1°C while petri dish relative humidity was 90%.

Data were collected after one month for callus fresh weight and number of embryos produced per each callus segment and number of regenerated shoots per non-each embryonic calli segment.

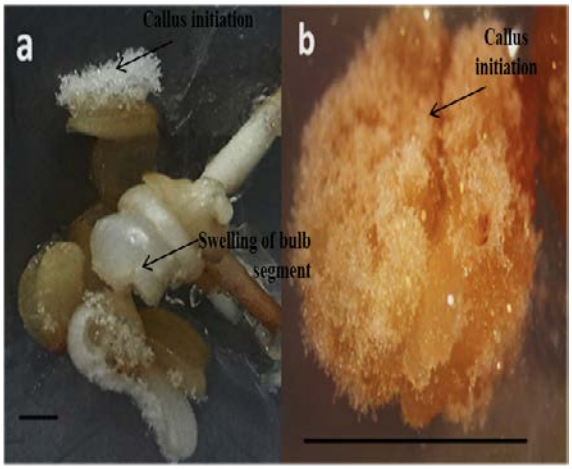

Figure 2. a) Start of callus initiation and swelling of *N. tazetta* explants cultured on MS media supplemented with 1.0 mg/L BAP and 2.0 2,4-D, and maintained under dark for 5 weeks. b) Callus initiation of *N. tazetta* explants cultured on MS media supplemented with 1.0 mg/L BAP and 2.0 2,4-D, and maintained under dark for 7 weeks. Scale bar = 1.0 cm.

2.3. Shoot development from embryos

Shoot development from the obtained embryos was experimented using MS media supplemented with (0.0, 0.2, 0.5, 1.0, 1.5, 2.0 mg l-1) of different growth regulators; (2iP, BAP, or KIN) plus 30 g sucrose. However, to induce shoot development, cultures were transferred from dark into a daily regime of 24±1°C under a 16/8 (light/dark) photoperiod of 45–50 µmol/ m2s irradiance. Numbers of regenerated shoots per segment of callus were recorded, and the percentage of germinated embryos was calculated. The regenerated shoots were subcultured into MS hormone-free media in 250 ml Erlenmeyer flasks. After rooting, developed plants were ready for acclimatization.

2.4. Acclimatization

Before acclimatization, *in vitro* produced planlets were maintained in MS hormone-free media for (2, 4 and 6 weeks) to increase bublet size before being transferred to greenhouse conditions, as increasing bublet size would increase survival chances for the plantlets during acclimatization *Ex vitro* acclimatization was performed for plantlets with well-developed roots. The plantlets were taken out form the flasks then the agar was washed away from the roots under running tap water, then the plantlets were cultured into sterilized plastic cups (5×5 cm) containing sterilized growing medium (peat: perlite mixture). Each cup was irrigated with distilled water every 3 days for 6 weeks. The potted plantlets were initially maintained inside the culture room conditions for 6 weeks and later transferred to green house (33 ±1°C) conditions for 8 weeks, and data were recorded for survival rate.

All conducted treatments were arranged in a completely randomized design (CRD). Data were statistically analyzed using SPSS, and analysis of variance (ANOVA) was used to analyze the obtained results; means

were separated with a probability level of 0.05 according to Tukey's honestly significant difference (HSD) test.

3. Result and discussion

3.1. Effect of plant growth regulators on somatic embryos

Embryo induction was observed after three weeks of inoculation of *N. tazetta* calli under concentrations of BAP and 2iP, while it took four weeks for embryos to appear in callus grown in KIN enriched media, while calli grown in the control treatment turned into brown and died. Using 2iP at 0.2 mg.l^{-1} was significantly the most effective treatment as it resulted in the highest values of callus weight (1818.25 mg), the number of somatic embryos (441.03/callus segment) and the number of regenerated shoots per non-embryonic calli (6.5) (Table 1, Figure 3). Meanwhile, high concentrations of 2iP had affected adversely callus growth, number of somatic embryos and regenerated shoots. Using 2iP for embryogenesis was reported in another study about *Iris nigricans* micropropagation, where embryos were successfully obtained in a medium supplemented with 1.0 mg l^{-1} 2iP as it yielded 2,686 embryos/g callus (Shibli and Ajlouni 2000). Similarly, Duquenne *et al.* (2006) in his study about "Zantedeschia hybrids reported that somatic embryos were regenerated into plantlets when cultured on MS medium supplemented with 1.0 mg l^{-1} 2iP. Meanwhile, our data showed that (KIN) at all used levels gave the least numbers of somatic embryos compared to the other plant growth regulators tested (Table 1). This result agrees with Shibli *et al.*, (2012), who found that KIN was the least effective cytokinin for the production of embryogenic callus of *Arum palaseitinum.*

Table 1: Effect plant growth regulators type and level on embryonic callus weight, the approximate number of somatic embryos/ callus segment and the number of regenerated shoots per non-embryonic calli of *N. tazetta* cultured on MS media in dark at growth room conditions for 4 weeks.

Growth regulator (mg/L)	Embryonic callus weight (mg)	Approximate number of somatic embryos/callus segment	Number of regenerated shoots/ nonembryonic calli segment
BAP			
C[x]	0.0 d [z]	0.0 d	0.0 a
0.2	1010 ab	262.6 ab	1.2 a
0.5	1256 a	326.56 a	1.8 a
1.0	710 c	184.6 c	0.8 a
1.5	730 bc	189.8 bc	1.4 a
2.0	546 c	141.96 c	1.8 a
2iP			
C[x]	0.0 d[z]	0.0 d	0.0 c
0.2	1818.25 a	441.03 a	6.5 a
0.5	1195.8 b	298.95 b	4.4 b
1.0	1201.60 b	300.4 b	1.6 c
1.5	952.50 bc	238.125 bc	1.33 c
2.0	669.4 c	167.35 c	1.0 c
KIN			
C[x]	0.0 c[z]	0.0 c	0.0 b
0.2	558.75 b	55.875 b	0.0 b
0.5	754 ab	82.94 ab	2.4 ab
1.0	670 ab	87.10 ab	0.4 b
1.5	825 ab	90.75 a	3.4 a
2.0	900 a	99.0 a	1.6 ab

[x] Control treatment represents hormone-free MS media. Each plant growth regulator was analyzed separately.[z] Means within columns having different letters are significantly different according to Tukey HSD at P≤0.05.

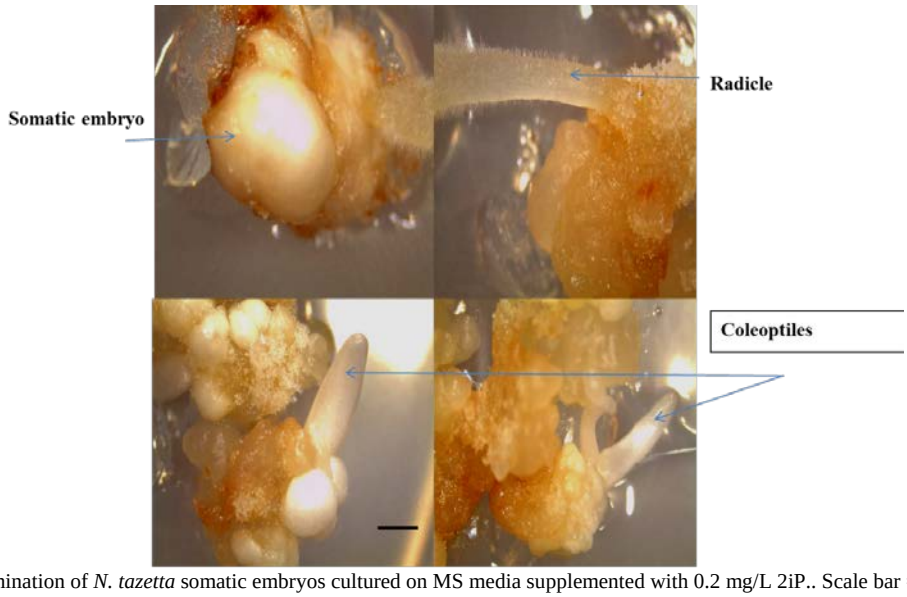

Figure 3. Germination of *N. tazetta* somatic embryos cultured on MS media supplemented with 0.2 mg/L 2iP.. Scale bar = 0.50 cm.

3.2. The effect of different carbon sources on somatic embryos

Our data revealed that sucrose at the level of 3% w/v was the best carbon source for N. *tazetta* when compared to fructose and glucose (Table 2). Meanwhile, 4% sucrose resulted in direct development of small bulblets and was not efficient for embryogenesis. On the other hand, glucose and fructose were not effective as a carbon source for embryos expression most callus formed became black with time and died (Table, 2). Sucrose was reported as the

best as a carbon source for micropropagation in many plant species, as it is the most popular carbohydrate in the plant phloem (Murashige & Skoog, 1962; Ahmad *et al*., 2007; Tahtamouni *et al*., 2016). Furthermore, in a study about strawberry, 6% sucrose was found superior not only for giving optimum embryo induction of embryonic culture but also a uniform embryo developmental stages compared to the other tested sugars (glucose and fructose), (Gerdakaneh *et al*., 2009).

Table 2. Effect of carbon sources and levels on embryonic callus weight, number of somatic embryos, and number of regenerated shoots from non-embryonic calli of *N. tazetta* calli cultured on MS media supplemented with 0.2 mg l^{-1} 2iP in dark at growth room conditions for 4 weeks.

Carbon source (g/L)	Embryonic callus weight (mg)	Approximate number of somatic embryos/callus segment	Number of regenerated shoots/ non-embryonic calli segment
Sucrose			
C[x]	0.0* c[z]	0.0 c	0.0 c
10	558.00 b	14.2 bc	3.20 ab
30	1818.25 a	441.03 a	6.5 a
40	520 b	39.8 b	4.4 a
50	520 b	16.7 bc	1.4 ab
Fructose			
C[x]	0.0 b[z]	0.0 b	0.0 a
10	483 a	7.7 a	0.4 a
30	446 a	0.0 b	0.0 a
40	465 a	0.0 b	0.0 a
50	473.6 a	0.0 b	0.0 a
Glucose			
C[x]	0.0 c[z]	0.0 b	0.0 a
10	289 b	0.0 b	0.2 a
30	381 b	13.5 a	0.8 a
40	625 a	2.2 b	0.0 a
50	289 b	0.8 b	0.0 a

[x] Control treatment represents sugar free MS media + 0.2 mg l^{-1} 2iP. Each sugar type was analyzed separately. [z] Means within columns having different letters are significantly different according to Tukey HSD at P≤0.05.

3.3. Shoot developmentdevelopment from embryos

The embryos started to germinate and developed into plantlets with shoots and roots after the subculture of embryonic callus into light conditions(Figure 4). Table 3 showed that 2iP at 0.5 mg.l^{-1} gave significantly the highest number of germinated shoots (191.99) and germination percentage (45.76 %) over the other concentrations or other plant growth regulators, while in BAP treatments, the highest number of germinated shoots (42.28) and germination percentage (16.3%) were obtained on MS media supplemented with 0.2 mg l^{-1}. Our data agree with, Lokhande *et al.* (2010) fiding on *Sesuvium portulacastrum* as the highest number of shoots, average shoot elongation, and percent shoot development per explant were observed on MS medium supplemented with 8.0 mg l^{-1} 2iP followed by 4.50 mg l^{-1} BAP. Moreover, *Zantedeschia* hybrids somatic embryos developed into plantlets on basal media supplemented with 1.0 mg l^{-1} 2iP (Duquenne *et al*., 2006). On the other hand, Shibli *et al.*, (2012) reported that the highest number of regenerated shoots from somatic embryos of *A. palaestinum* was achieved on MS media supplemented with 2.0 mg l^{-1} BAP

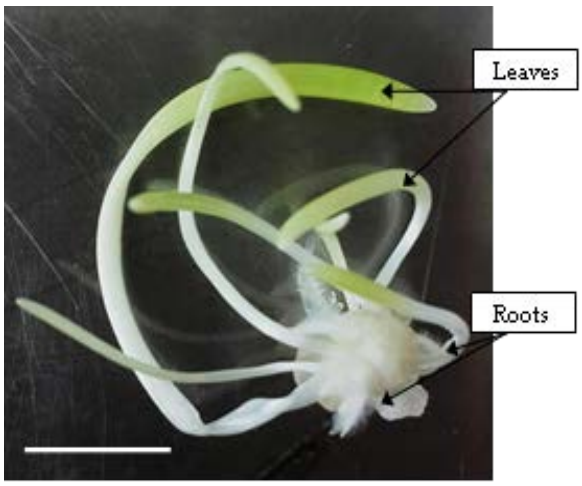

Figure 4. Regenerated *N. tazetta* plantlet from somatic embryos cultured on MS media supplemented with 0.5 mg/L of 2iP, and maintained under growth room conditions of 24±1°C under a 16/8 (light/dark) photoperiod of 45–50 µmol/ m2s irradiance and flask relative humidity of 90% for 4 weeks. Scale bar = 1.0 cm.

Table 3: Effect of plant growth regulators type and level on the number of germinated shoots from somatic embryos and germination percentage of *N. tazetta* somatic embryos cultured on MS media for 4 weeks.

Growth regulator (mg/L)	Number of germinated shoots from somatic embryos	Germination percentage of somatic embryos % (Number of germinated shoots/number of somatic embryos %)
BAP		
C[x]	0.0 c	0.0 d
0.2	42.28 a	16.3 a
0.5	16.02 b	4.95 b
1.0	5.28 c	2.76 bcd
1.5	3.56 c	1.84 cd
2	5.11 c	3.36 bc
2iP		
C[x]	0.0 c	0.0 d
0.2	78.95 b	29.44 b
0.5	191.99 a	45.76 a
1.0	51.24 b	16.32 c
1.5	11.95 c	5.12 d
2	5.30 c	3.20 d
KIN		
C[x]	0.0 c	0.0 c
0.2	0.0 c	0.0 c
0.5	10.90a	9.532ab
1.0	3.72 ab	3.70 bc
1.5	13.08 a	12.00 a
2	5.08 ab	5.08 abc

[x] Control represents free hormone MS media supplemented with 30 g/L sucrose. Each plant growth regulator was analyzed separately. [z]Means within columns having different letters are significantly different according to Tukey HSD at P≤0.05.

3.4 Ex Vitro Acclimatization

Our results showed that the period of incubating the plantlets on hormone-free MS media plus 30 g sucrose before acclimatization had positively affected bulb size measured after 5 weeks in the greenhouse conditions. Most plantlents incubated for 2 weeks in hormone-free MS media produced bulblets with approximately 0.5 - 0.6 cm in diameter and produced only a single leaf, while those inculcated for 4 weeks produced bulblets with 0.9 - 1.1 cm in diameter and 2 leaves. On the other hand plantlets inoculated for 6weeks produced bulblets with 1.4 - 1.6 cm in diameter and 3 leaves (Figure 5).

Meanwhile, most *in vitro* produced plants of *N. tazetta* L. showed excellent survival rate of 95% in growth room and 100% in the greenhouse. The acclimatized plants were normal and did not show any morphological abnormalities (Figure 6).

Figure 5. a) Bulblet of *N. tazetta* inculcated for 2 weeks on hormone-free MS media with approximately 0.5 cm diameter and single leaf. b) Bulblet of *N. tazetta* inculcated for 4 weeks on hormone-free MS media with approximately 1.0 cm diameter and two leaves. c) Bulblet of *N. tazetta* plant inculcated for 6 weeks on hormone-free MS media with approximately 1.6 cm diameter and three leaves. Scale bar = 1.0 cm.

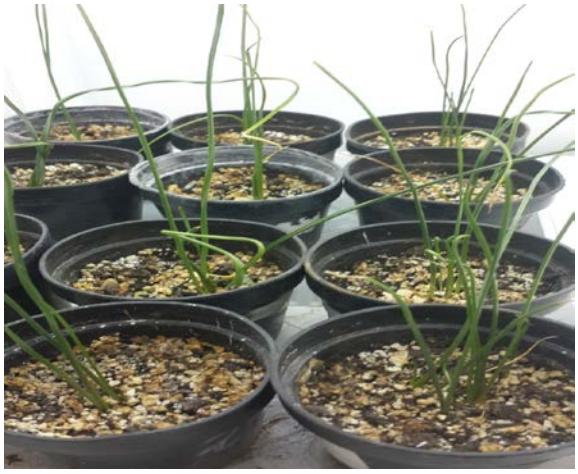

Figure 6. Successful acclimatization of *N. tazetta* plants maintained for 5 weeks on 1:1 peat perlite medium under greenhouse conditions and irrigated with tap water every 3 days with a 95% survival rate.

4. Conclusion

The current protocol could represent a successful tool for rapid micropropagation of *N. tazetta* via somatic embryogenesis using MS media + 0.2 mg/L2iP + 30 g sucrose. Meanwhile, adding 0.5 mg/L 2iP + 30 g sucrose to the culture media was required for maximum shoot development from the resulting embryos. This protocol could be applied commercially to produce a high number of plants with high survival rates. Further studies can be done to produce flowering size bulbs that can flower in the current or next season. Also, more research can be conducted to induce production of medicinally important secondary metabolites including galantamine (GAL) in *N. tazetta* plant cells *in vitro*.

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